

Programming of enzyme specificity by substrate mimetics: investigations on the Glu-specific V8 protease reveals a novel general principle of biocatalysis

Nicole Wehofskey, Frank Bordusa*

Department of Biochemistry, Faculty of Biosciences, Pharmacy and Psychology, University of Leipzig, TalstraÙe 33, D-04103 Leipzig, Germany

Received 10 November 1998; received in revised form 12 December 1998

Abstract In this paper the universal validity of the substrate mimetic concept in enzymatic C-N ligations was expanded to anionic leaving groups based on the specificity determinants of Glu-specific endopeptidase from *Staphylococcus aureus* (V8 protease). In an empirical way a specific mimetic moiety was designed from simple structure-function relationship studies. The general function of the newly developed substrate mimetics to serve as an artificial recognition site for V8 protease have been examined by hydrolysis kinetic studies. Enzymatic peptide syntheses qualify the strategy of substrate mimetics as a powerful concept for programming the enzyme specificity in the direction of a more universal application of enzymes in the general area of biocatalysis.

© 1999 Federation of European Biochemical Societies.

Key words: Endoproteinase Glu-C (V8 protease); Substrate mimetic; Acyl transfer; Enzyme specificity; Peptide synthesis

1. Introduction

It has been demonstrated that peptide synthesis catalyzed by native proteases and special mutants is more advantageous than chemical synthesis in many respects [1–3]. The enzymatic formation of peptide bonds is highly regio- and stereospecific and, therefore, does not require large experimental efforts to protect side chains of trifunctional amino acids. Moreover, chemical peptide synthesis of large peptides is often difficult because of the accumulation of side products that complicate product purification and decrease yields [4,5]. Additionally, coupling of peptide fragments by selective chemical ligation strategies is limited to special N-terminal amino acids in the amino component, i.e. Cys, Thr or Ser [6–10]. For this reason, the development of enzyme-based alternatives for selective peptide bond formations, especially for fragment condensations, is a useful challenge since a native C-N peptide ligase was not developed in evolution.

Apart from the undisputed advantages, the application of the enzymatic approach is limited both by the restricted substrate specificity of proteases and the risk of undesired proteolysis of reactants and peptide products. To overcome these

limitations the application of a new type of acyl donors, formerly called inverse substrates, for trypsin was reported [11–17]. Recently, we described an extension of this approach for other trypsin-like proteases and the term substrate mimetics was introduced [18].

This kind of substrate, originally developed as time-dependent irreversible inactivators of trypsin and trypsin-like enzymes [19], characteristically bears an ester leaving group, i.e. the 4-guanidino- or 4-amidinophenyl functionality which interacts with the active site of these proteases in a way that mimics specific arginine and lysine side-chains of trypsin or trypsin-like enzymes (Fig. 1). Previously, by theoretical docking simulations we could further demonstrate that the leaving group moiety of these substrates binds in place of the specificity-determining basic side-chain functionality of common acyl donors largely independently of its C-terminal amino acid [18]. As a result, peptide coupling occurs irreversibly and independently of primary specificity of these enzymes. Moreover, the molecular dynamic calculations imply a novel, general principle in biocatalysis that should not only be restricted to cationic specificity determinants derived from trypsin and trypsin-like proteases.

Based on these new findings endoproteinase Glu-C (V8 protease; EC 3.4.21.19) from *Staphylococcus aureus* was chosen to test the universal validity of the substrate mimetic concept for programming the specificity of a protease. Firstly, in an empirical way a new type of substrate mimetics bearing anionic specificity determinants was developed and the specificity constants of these new substrates were determined. Because of the restrictive substrate specificity, i.e. for the hydrolysis of peptide bonds at the carboxyl side of Glu in preference to Asp, V8 protease is a suitable biocatalyst for condensation of peptide fragments, recently shown by the synthesis of an analog of human growth hormone releasing factor [20]. To clarify substrate mimetics as powerful tools in biocatalysis the newly developed substrate mimetics were used as acyl donor components in V8 protease-catalyzed peptide bond formations. Finally, the successful application of this strategy for model peptide segment condensations could be demonstrated.

2. Materials and methods

2.1. Materials

V8 protease (EC 3.4.21.19) was obtained from Fluka Chemie AG, Switzerland and had a specific activity of 800 U/mg (1 U=amount of enzyme that hydrolyzes 1 µmol/min *N*-Boc-L-Glu-α-phenyl ester at pH 7.8, 37°C). Amino acids and peptide derivatives were purchased from Bachem, Switzerland, Serva, Germany or Degussa AG, Germany. If not otherwise stated, all reagents were of the highest available commercial purity. Solvents were purified and dried by usual methods.

*Corresponding author. Fax: (49) (341) 9736998.

E-mail: bordusa@uni-leipzig.de

Abbreviations: a, amide; Boc, *tert*-butoxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; NEM, 4-ethylmorpholine; SCam, carboxamidomethyl thioester; SCm, carboxymethyl thioester; SME, methyl thioester; Xaa, amino acid residue; Z, benzyloxycarbonyl

Dedicated to Prof. Dr. H.-D. Jakubke on the occasion of his 65th birthday.

2.2. Synthetic chemistry

Z-protected amino acid and peptide carboxymethyl and carboxamidomethyl thioesters were synthesized by coupling of the appropriate N $^{\alpha}$ -protected amino acid or peptide with thioglycolic acid and thioglycolic acid amide using the mixed anhydride method (isobutylchloroformate/NEM). In the same way Z-Glu-SMe was synthesized starting from Z-Glu(Boc)-OH and sodium methanethiolate. The penta- and decapeptides Ile-Ala-Ala-Ala-Gly and Leu-Ala-Phe-Ala-Lys-Ala-Asp-Ala-Phe-Gly were synthesized with a semiautomatic batch peptide synthesizer SP 650 (Labortechnik AG, Switzerland) using *p*-alkoxybenzyl alcohol resin, synthesized according to Wang [21] and standard Fmoc chemistry. The peptides were precipitated with dry diethyl-, diisopropylether or mixtures of hexane/ethylacetate. The identity and purity of all peptides and substrate esters were characterized by analytical HPLC, NMR, thermospray mass spectroscopy and elementary analysis. In all cases satisfactory analytical data ($\pm 0.4\%$ for C, H, N) were found.

Z-L-Ala-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 1.27 (d, 3H), 3.64 (s, 2H), 4.22 (m, 1H), 5.08 (s, 2H), 7.35 (m, 5H), 8.13 (d, 1H), 13.01 (s, 1H).

Z-D-Ala-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 1.27 (d, 3H), 3.65 (s, 2H), 4.23 (m, 1H), 5.08 (s, 2H), 7.35 (m, 5H), 8.13 (d, 1H), 13.01 (s, 1H).

Z-L-Phe-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 3.12 (m, 2H), 3.67 (s, 2H), 4.39 (m, 1H), 5.01 (s, 2H), 7.26 (m, 5H), 8.21 (d, 1H), 13.01 (s, 1H).

Z-L-Ser-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 3.64 (d, 2H), 3.68 (s, 2H), 4.25 (m, 1H), 4.91 (s, 1H), 5.05 (s, 2H), 7.35 (m, 5H), 7.95 (d, 1H), 12.75 (s, 1H).

Z-L-Pro-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 1.88 (m, 7H), 3.65 (s, 2H), 4.02 (m, 1H), 5.06 (s, 2H), 7.34 (m, 5H), 12.98 (s, 1H).

Z-Gly-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 3.67 (s, 2H), 3.95 (d, 2H), 5.05 (s, 2H), 7.35 (m, 5H), 8.06 (t, 1H), 13.01 (s, 1H).

Z-L-Met-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 1.87 (m, 2H), 1.91 (m, 2H), 2.02 (s, 3H), 3.64 (s, 2H), 4.32 (m, 1H), 5.06 (s, 2H), 7.35 (m, 5H), 13.01 (s, 1H).

Z-Pro-Leu-Gly-SCm. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 0.75 (m, 3H), 0.86 (m, 3H), 1.42 (m, 1H), 1.56 (m, 2H), 1.82 (m, 7H), 3.64 (s, 2H), 3.98 (d, 2H), 4.21 (m, 1H), 5.08 (s, 2H), 7.33 (m, 5H), 8.05 (t, 1H), 8.14 (d, 1H), 13.00 (s, 1H).

Z-L-Phe-SCam. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 3.10 (m, 2H), 3.55 (s, 2H), 4.36 (m, 1H), 5.0 (s, 2H), 7.31 (m, 10H), 7.53 (s, 2H), 8.20 (d, 1H).

Z-L-Glu-SMe. $^1\text{H-NMR}$ (300 MHz, DMSO) δ 0.87 (s, 3H), 1.96 (m, 4H), 4.18 (m, 1H), 5.04 (s, 2H), 7.34 (m, 5H), 8.05 (d, 1H), 12.10 (s, 1H).

2.3. Enzyme kinetics

Hydrolysis reactions were performed in a total volume of 500 μl containing 0.2 M HEPES buffer, pH 8.0 at 37°C. After thermal equilibration of assay mixtures the reactions were started by the addition of 10 μl of enzyme stock solutions resulting in enzyme concentrations of 0.003–9.9 μM . At defined time intervals 50 μl was withdrawn and diluted with 50 μl stop solution containing 50% methanol and 5% trifluoroacetic acid. The stability of the thioesters under this acidic conditions was examined by reanalysis of samples of each ester after storage of several days. The rate of reaction was analyzed by HPLC determining the disappearance of substrate esters. An experiment without enzyme was carried out to quantify the extent of non-enzymatic ester hydrolysis which was strictly less than 3%. The substrate concentrations were 0.01–3.0 mM. Within this range no saturation of

the enzyme by the substrates could be observed. The kinetic data reported were calculated from the linear slopes of the initial Michaelis-Menten curve in relation to the appropriate enzyme concentration using the software SigmaPlot Scientific Graphic System (Vers. 1.01, Jandel Corp., USA).

2.4. Acyl transfer studies

Enzymatic reactions were performed in a total volume of 250 μl containing 0.2 M HEPES buffer, pH 8.0 at 37°C. Stock solutions of acyl donor esters (4 mM) and nucleophiles (40 or 20 mM) were prepared in 0.2 M HEPES buffer, pH 8.0. To the stock solutions of the nucleophilic components appropriate equivalents of NaOH were added to neutralize hydrochlorides or hydrobromides. The final acyl donor concentration was 2 mM and the final nucleophile concentration was 20 or 10 mM. The latter was calculated as free, N $^{\alpha}$ -unprotonated nucleophile concentration $[\text{HN}]_0$ according to the formalism of Henderson-Hasselbalch:

$$[\text{HN}]_0 = \frac{[\text{N}]_0}{1 + 10^{pK_{\alpha} - \text{pH}}} \quad (1)$$

The pK_{α} values of the α -amino group of the nucleophiles were determined by inflection point titration on a Video-Titrator VIT 90 (Radiometer, Denmark). Measurements were carried out at a nucleophile concentration of 6.7 mM using 0.1 M HCl as titrant. The pK_{α} values were calculated by linear interpolation from the slope minimum range. S.D. was estimated to be $pK_{\alpha} \pm 0.01$. After thermal equilibration of the assay mixture the enzymatic coupling reactions were initiated by the addition of 7 μl of enzyme stock solutions leading to enzyme concentrations of 0.1–4.0 μM . At certain time intervals 50 μl was withdrawn and diluted with 50 μl stop solution containing 50% methanol and 5% trifluoroacetic acid. Reaction times of 24 h led to an ester consumption of 70–100%. For each substrate and nucleophile an experiment without enzyme was carried out to determine the extent of non-enzymatic ester hydrolysis which was strictly less than 10%. Based on the same controlled experiments non-enzymatic aminolysis of the thioester was investigated and could be ruled out. The identity of the formed peptide products was established by thermospray mass spectroscopy.

2.5. Segment condensations

Enzymatic segment condensations were performed as described above. To realize a complete solubility of the decapeptide the assay mix contained 5% DMSO. The reaction products were identified by MALDI-TOF mass spectroscopy.

Z-Pro-Leu-Gly-Ile-Ala-Ala-Ala-Gly-OH. MALDI-TOF calculated for $\text{C}_{38}\text{H}_{58}\text{N}_8\text{O}_{11} + \text{Na}^+$ 802.94, found 802.92.

Z-Pro-Leu-Gly-Leu-Ala-Phe-Ala-Lys-Ala-Asp-Ala-Phe-Gly-OH. MALDI-TOF calculated for $\text{C}_{69}\text{H}_{98}\text{N}_{14}\text{O}_{18} + \text{Na}^+$ 1411.55, found 1411.48.

2.6. HPLC analysis

HPLC measurements were performed using a Shimadzu LC-10A HPLC system and analyzed with Shimadzu LC-10 software (Japan). A Lichrospher RP 18 column (250 \times 4 mm, 5 μm , Merck, Germany) was used. Samples were eluted under isocratic conditions with various mixtures of acetonitrile and water containing 0.1% trifluoroacetic acid at flow rates of 0.6–1.0 ml/min. All measurements were detected at 254 nm. The product yields were calculated from peak areas of ester substrates, hydrolysis and aminolysis products. In the case of the decapeptide which contains additional chromophoric amino acid residues the product yields were calculated from the lack of hydrolysis

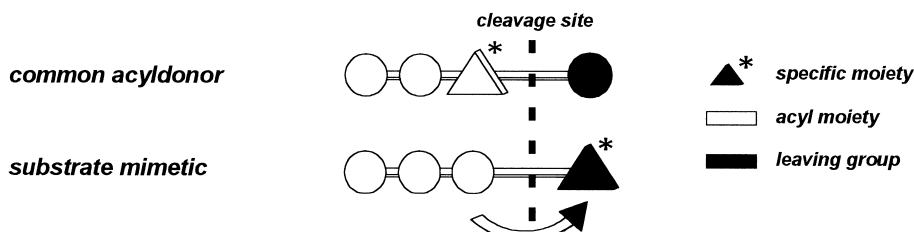


Fig. 1. Schematic structures of common acyl donor components versus reversed-type substrate mimetics.

Table 1

Kinetic parameters for V8 protease-catalyzed hydrolysis of carboxymethyl thioester substrate mimetics compared to the normal-type substrate Z-Glu-SMe

Substrate	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
Z-Glu-SMe	1.12×10^4
Z-Ala-SCm	1.58×10^1
Z-D-Ala-SCm	0.97×10^1
Z-Phe-SCm	2.01×10^1
Z-Ser-SCm	0.87×10^1
Z-Pro-SCm	0.13×10^1
Z-Gly-SCm	3.34×10^1
Z-Met-SCm	4.60×10^1
Z-Pro-Leu-Gly-SCm	3.18×10^2
Z-Phe-SCam	no hydrolysis

Conditions: 0.2 M HEPES, pH 8.0, 37°C, [substrate]: 0.0125–3 mM, [enzyme]: 0.003–9.9 μM ; errors are less than 15%.

product at the end of reactions using 4-nitrophenol as internal standard.

3. Results and discussion

3.1. Design of a novel substrate mimetic type

As shown by our docking studies on the trypsin-type model the leaving group moiety of a substrate mimetic binds in place of the specificity-determining amino acid side chain of common substrates [18]. Therefore, an important condition for this function is a high affinity of the leaving group to the primary substrate specificity of the appropriate enzyme, i.e. to the strong Glu preference of V8 protease at the S_1 position (nomenclature according to [22]). Unfortunately, the unknown X-ray structure of this enzyme allows only the design of suitable mimetic moieties by empirical structure-function relationship studies based on the known native enzyme specificity. In this way the carboxymethyl thioester functionality was selected as a potentially suitable leaving group for imitating specific Glu residues. In order to investigate the features of the corresponding acyl carboxymethyl thioesters for V8 protease-catalyzed reactions a variety of N^α -protected amino acid and peptide esters were synthesized.

3.2. Enzyme kinetics

The general function of the carboxymethyl thioester group to serve as a new artificial recognition site for V8 protease was examined by steady-state hydrolysis kinetic studies. To eval-

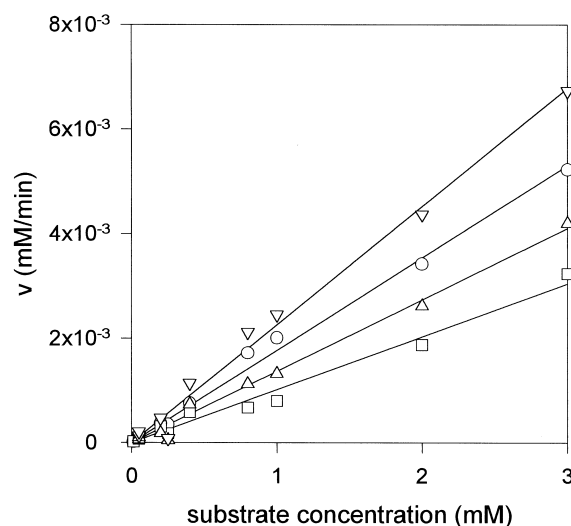


Fig. 2. Kinetics of V8 protease-catalyzed hydrolysis of carboxymethyl thioesters bearing non-specific acyl moieties compared to the normal type substrate Z-Glu-SMe. (∇) Z-Gly-SCm, (\circ) Z-Ala-SCm, (\triangle) Z-Gly-SMe, (\square) Z-D-Ala-SCm. Conditions: 0.2 M HEPES, pH 8.0, 37°C. [substrates]: 0.0125–3 mM, [enzyme]: 0.003–9.9 μM .

uate the mimic efficiency of the carboxylate functionality of these esters the hydrolysis of the neutral carboxamidomethyl thioester Z-Phe-SCam was investigated. As a point of reference the common substrate Z-Glu-SMe was used. Plots of the initial rates of hydrolysis for some substrates are shown in Fig. 2. As indicated by the straight lines of these plots in all cases no saturation of the enzyme by the substrate, including the normal-type substrate ester, could be observed. The resulting specificity constants (k_{cat}/K_m) calculated from the linear slopes of the corresponding curves are listed in Table 1. Generally, the carboxymethyl thioester functionality was found to mediate a specific hydrolysis of all substrates independently of its C-terminal amino acid V8 protease which also holds for the amino acid Pro and even for the non-coded D-conformer of Ala. Remarkably, D-Ala causes only a slight decrease in specificity compared to the L-counterpart. In contrast to this non-specificity of the enzyme for the acyl residue, the negative charge of the leaving group is essential to mimic substrates. Accordingly, the lack of this charge in the case of Z-Phe-SCam causes a complete loss of specificity. Analyzing the

Table 2

Partition values of V8 protease-catalyzed acyl transfer reactions of carboxymethyl thioester substrate mimetics with amino acid amides compared to Z-Glu-SMe

Acyl donor	Partition value (mM)			
	Acyl acceptor			
	H-Leu-NH ₂	H-Ile-NH ₂	H-Nva-NH ₂	H-Met-NH ₂
Z-Glu-SMe	18.4	26.6	29.5	49.5
Z-Ala-SCm	17.2	18.7	20.0	22.8
Z-D-Ala-SCm	40.4	35.9	44.2	42.5
Z-Phe-SCm	3.90	5.40	6.90	12.8
Z-Ser-SCm	32.9	20.1	28.5	33.8
Z-Pro-SCm	78.1	82.1	101	108
Z-Gly-SCm	28.9	27.4	33.7	36.7
Z-Met-SCm	37.9	23.1	36.4	52.8
Z-Pro-Leu-Gly-SCm	28.8	31.9	29.0	35.6

Conditions: 0.2 M HEPES, pH 8.0, 37°C, [acyl donor]: 2 mM, [acyl acceptor]: 20 mM, [enzyme]: 0.3–4 μM ; errors are less than 15%.

Table 3

Partition values of V8 protease-catalyzed acyl transfer reactions of carboxymethyl thioester substrate mimetics and dipeptide amides compared to Z-Glu-SMe

Acyl donor	Partition value (mM)		
	Acyl acceptor		
	H-Leu-Ala-NH ₂	H-Leu-Gly-NH ₂	H-Gly-Leu-NH ₂
Z-Glu-SMe	7.10	3.42	50.0
Z-Ala-SCm	12.9	17.6	21.8
Z-D-Ala-SCm	20.3	28.3	30.5
Z-Phe-SCm	4.00	8.11	15.7
Z-Ser-SCm	31.5	13.9	16.4
Z-Pro-SCm	139	92.2	93.7
Z-Gly-SCm	38.4	22.5	48.0
Z-Met-SCm	38.6	12.1	63.0

Conditions: 0.2 M HEPES, pH 8.0, 37°C, [acyl donor]: 2 mM, [acyl acceptor]: 20 mM, [enzyme]: 0.3–4 µM; errors are less than 15%.

specificity constants for the carboxymethyl thioesters a one to four orders of magnitude lower specificity compared to the common substrate was found. Interestingly, the elongation of the peptide chain in the case of Z-Pro-Leu-Gly-SCm increases the specificity by one order of magnitude compared to the shorter counterparts resulting in a 35-fold lower $k_{\text{cat}}/K_{\text{m}}$ value than found for Z-Glu-SMe. Therefore, the acyl carboxymethyl thioesters qualify as specific substrate mimetics for V8 protease-catalyzed hydrolysis.

3.3. Deacylation kinetics

In the presence of added nucleophilic amino components, cysteine and serine proteases catalyze the acyl transfer to nucleophiles simultaneously with the hydrolyses of the acyl donors. Consequently, the resulting hydrolysis product Ac-OH, which possesses a very low acylation potential, and the resulting peptide product Ac-N are formed. As an efficiency parameter of both competitive reactions the partition value p was introduced analogously to the definition of the Michaelis constant K_{m} [23].

$$p = \frac{d[\text{Ac} - \text{OH}]}{d[\text{Ac} - \text{N}][\text{HN}]} = \frac{k_3 K_n}{k_4} + \frac{k_5}{k_4} [\text{HN}] = p_0 + p_n [\text{HN}] \quad (2)$$

According to Eq. 2 the p value is defined as the nucleophile concentration at which the rates of both reactions are equal (corresponds to 50% yield). Consequently, a decrease in the p value is oppositely correlated with an increase in peptide yield. Using nucleophile excess the partition value can be determined from the product ratios according to:

$$p = \frac{[\text{Ac} - \text{OH}]}{[\text{Ac} - \text{N}][\text{HN}]} \quad (3)$$

where [HN] is the initial nucleophile concentration and [Ac-OH] and [Ac-N] represent the product concentrations.

3.4. Acyl transfer studies

The basic features of carboxymethyl thioesters for V8 protease-catalyzed peptide bond formations were characterized by analytical acyl transfer reactions. For this purpose the newly developed substrates listed in Table 1 served as acyl donor components in simple model reactions using amino acid and dipeptide amides as acyl acceptors. The resulting partition values of coupling reactions are summarized in Table 2 for amino acid amides and Table 3 for dipeptide amides.

Generally, each carboxymethyl thioester is able to serve as an acyl donor for V8 protease-catalyzed peptide synthesis independently of its acyl moiety which has been predicted by the hydrolysis kinetic studies. Analyzing the data shown in Tables 2 and 3, in most cases the p values observed for the substrate mimetics are comparable to that of the normal-type acyl donor Z-Glu-SMe. Therefore, in spite of the greatest partition values found for the proline acyl donor ester which also shows the lowest specificity constant, no correlation between substrate specificity and efficiency of peptide bond formation is evident. Moreover, if dipeptide amides were used as acyl acceptors mostly lower p values were found for substrate mimetics than for Z-Glu-SMe. These findings imply a yield-increasing effect of longer acyl acceptors, especially when carboxymethyl thioesters serve as acyl donors. As a result these substrate mimetics should be suitable acyl donors for V8 protease-catalyzed segment condensations.

3.5. Segment condensations

The data reported in Fig. 3 confirm the suggestion mentioned above. Consequently, for the penta- and decapeptide for both acyl acceptors, lower p values and therefore higher product yields could be obtained than found for the small dipeptide amides whereas the decapeptide is more effective than the pentapeptide. A further advantage of substrate mimetic-mediated reactions results from the non-specificity of the coupled amino acid residue. In contrast to enzymatic reac-

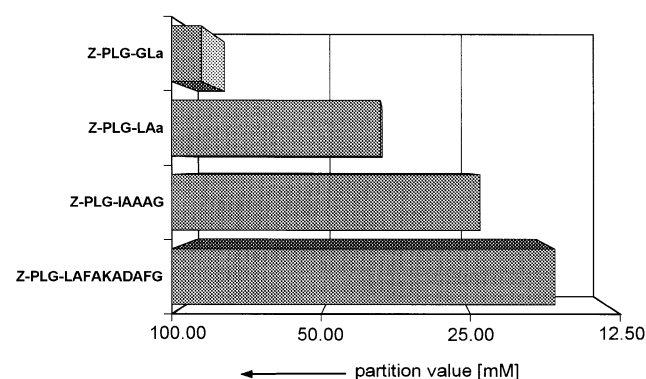


Fig. 3. Partition values of V8 protease-catalyzed peptide couplings using Z-Pro-Leu-Gly-SCm as acyl donor. Conditions: 0.2 M HEPES, pH 8.0, 37°C, [acyl donor]: 2 mM, [LAFAKADAFG]: 10 mM, [dipeptide amides, IAAAG]: 20 mM, [enzyme]: 4.9 µM.

tions using normal-type acyl donors, once formed the peptide bond reached from substrate mimetics cannot be cleaved by the enzyme again. Universal application of this strategy may only be limited when using specific amino acid containing reagents, i.e. Glu-containing peptides. In these cases the Glu residue should be placed at non-cleavable positions (e.g. on the C-terminus of the amino component) to avoid competitive proteolytic side reactions in the starting material.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (JA 559/9-1, INK 23) and the Fonds der Chemischen Industrie. We would like to thank Prof. H.-D. Jakubke for inspiring discussions and Prof. P. Halling, Strathclyde University, Glasgow, for reading and correcting the manuscript. The authors thank ASTA Medica AG and Fluka AG for special chemicals.

References

- [1] Schellenberger, V. and Jakubke, H.-D. (1991) *Angew. Chem. Int. Ed. Engl.* 30, 1437–1449.
- [2] Wong, C.-H. (1989) *Science* 244, 1145–1152.
- [3] Jackson, D.Y., Burnier, J., Quan, C., Stanley, M., Tom, J. and Wells, J.A. (1994) *Science* 266, 243–247.
- [4] Bodanszky, M. and Bodanszky, A. (1984) *The Practice of Peptide Synthesis*, Springer-Verlag, New York.
- [5] Kaiser, E.T. (1989) *Acc. Chem. Res.* 57, 957.
- [6] Kemp, D.S., Grattan, J.A. and Reczek, J. (1975) *J. Org. Chem.* 40, 3465–3466.
- [7] Fotouhi, N., Galakatos, N.G. and Kemp, D.S. (1989) *J. Org. Chem.* 54, 2803–2809.
- [8] Schnölzer, M. and Kent, S.B.H. (1992) *Science* 256, 221–225.
- [9] Dawson, P.E., Muir, T.M., Clark-Lewis, I. and Kent, S.B.H. (1994) *Science* 266, 776–779.
- [10] Liu, C.-F. and Tam, J.P. (1994) *Proc. Natl. Acad. Sci. USA* 91, 6584–6588.
- [11] Tanizawa, K., Kasaba, Y. and Kanaoka, Y. (1977) *J. Am. Chem. Soc.* 99, 4484–4488.
- [12] Schellenberger, V., Jakubke, H.-D., Zapevalova, N.P. and Mitin, Y.V. (1991) *Biotechnol. Bioeng.* 38, 104–108.
- [13] Schellenberger, V., Jakubke, H.-D., Zapevalova, N.P. and Mitin, Y.V. (1991) *Biotechnol. Bioeng.* 38, 319–321.
- [14] Mitin, Y.V., Schellenberger, V., Schellenberger, U., Jakubke, H.-D. and Zapevalova, N.P. (1990) in: *Peptides* (Giralt, E. and Andreu, D., Eds.), pp. 287–288, ESCOM, Leiden.
- [15] Sekizaki, H., Itoh, K., Toyota, E. and Tanizawa, K. (1996) *Chem. Pharm. Bull.* 44, 1577–1579.
- [16] Sekizaki, H., Itoh, K., Toyota, E. and Tanizawa, K. (1996) *Chem. Pharm. Bull.* 44, 1585–1587.
- [17] Sekizaki, H., Itoh, K., Toyota, E. and Tanizawa, K. (1997) *Tetrahedron Lett.* 38, 1777–1780.
- [18] Bordusa, F., Ullmann, D., Elsner, C. and Jakubke, H.-D. (1997) *Angew. Chem. Int. Ed. Engl.* 36, 2473–2475.
- [19] Wagner, G. and Horn, H. (1973) *Pharmazie* 28, 428–431.
- [20] Bongers, J., Liu, W., Lambros, T., Breddam, K., Campbell, R.M., Felix, A.M. and Heimer, E.P. (1994) *Int. J. Peptide Protein Res.* 44, 123–129.
- [21] Wang, S.S. (1973) *J. Am. Chem. Soc.* 95, 1328–1333.
- [22] Schechter, I. and Berger, A. (1967) *Biochem. Biophys. Res. Commun.* 27, 157–167.
- [23] Könnecke, A., Schellenberger, V., Hofmann, H.-J. and Jakubke, H.-D. (1984) *Pharmazie* 39, 785–786.